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# Human but not rat amylin shares neurotoxic properties with Aβ42 in long-term hippocampal and cortical cultures

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**Abstract Type 2 diabetes mellitus (DM) and Alzheimer's disease (AD) share epidemiological and biochemical features. Both are characterized by insoluble protein aggregates with a fibrillar conformation – amylin in Type 2 DM pancreatic islets, and Aβ in AD brain. To determine whether amylin shares neurotoxic properties with Aβ, we incubated hippocampal and cortical neurons with Aβ42 and human amylin. Different from non-amyloidogenic rat amylin, both caused a dose-, time- and cell type-specific neurotoxicity supporting the notion of a similar toxic mechanism. Depending on the cell type, this finding is also supported by co-incubation of human amylin and Aβ.**



*Keywords:* Aβ42 amyloid; Alzheimer's disease; Amylin; Cell-type specificity; Conformational disease; IAPP; Neurotoxicity; Type 2 diabetes mellitus

## 1. Introduction

Type 2 diabetes mellitus (DM) and Alzheimer's disease (AD) are chronic diseases and both are leading causes of morbidity and mortality in the elderly. Type 2 DM accounts for 90% of all cases of diabetes and is characterized by resistance of target tissue to insulin and subsequently, elevated blood glucose levels. Disease progression is associated with increased deposition of amyloid-like amylin, also known as the islet amyloid polypeptide (IAPP), in insulin-producing pancreatic islet β-cells [1]. Amylin is a member of structurally and functionally related peptides which include calcitonin, adrenomedullin and calcitonin gene related peptide [2]. In humans, IAPP is synthesized as a 67-residue propeptide and colocalized with insulin in β-cell granules. The mature 37-amino acid peptide is produced by proteolysis (Fig. 1). Both in vitro and in vivo studies revealed that its formation is associated with β-cell death [1,3]. Type 2 DM can be defined as a conformational disease because amylin undergoes a change in tertiary structure followed by self-association and deposition [4]. Type 2 DM has been modelled by injecting streptozotocin [5] and in the ob/ob (leptin knockout), db/db (leptin receptor knockout), both of which are insulin resistant, and in mice with transgenic over-expression of IAPP in pancreatic islets [6–8]. Breeding the IAPP transgenic mice to homozygosity causes islet amylin aggregation, β-cell death and DM [8]. In conclusion, Type 2 DM is an amyloidosis and current evidence suggests a major, yet poorly understood, role of amylin in disease.

AD, for comparison, is a progressive neurodegenerative disease affecting more than 15 million people worldwide. It is characterized by progressive loss of memory and other cognitive functions, resulting in dementia. Like Type 2 DM, AD can also be defined as a conformational disease. In AD brain, the Aβ peptide and the protein tau undergo a change in tertiary structure followed by self-association and deposition in brain [9]. Aβ is the major constituent of β-amyloid plaques derived from the precursor protein APP, with Aβ42 being more fibrillogenic than Aβ40. Hyperphosphorylated forms of the protein tau are major constituents of neurofibrillary tangles (NFTs) [10]. In familial cases of AD, mutations were identified in the APP gene itself and in genes encoding APP proteases. In frontotemporal dementia (FTD), mutations were identified in tau [11–13]. We, along with others, utilized this information to develop transgenic animal models and achieved Aβ plaque and NFT formation [13,14]. Significant neuronal cell loss was found upon massive over-expression of FTD mutant tau [15]. Protein aggregates (such as Aβ and tau) characterize AD and FTD, but how Aβ and tau cause nerve cell dysfunction and degeneration is still poorly understood [16].

Aβ and human amylin are similar in size. They share little similarities in their primary sequence (Fig. 1), but interestingly, they fold into similar secondary structures. There is multiple evidence that they may exert toxicity by binding to the same receptor as, for example, antagonists of the amylin receptor such as AC187 block Aβ toxicity in rat cholinergic basal forebrain neurons [17]. Furthermore, both Aβ and human amylin can cause increased levels of APP, a putative Aβ receptor, in primary neuronal and astrocyte cultures [18]. Finally, distinct integrin signalling pathways mediate both Aβ- and amylin-induced neurotoxicity, and both can be inhibited with integrin-specific antibodies and cytochalasin D [19]. In support of the notion that it is the tertiary rather than the primary structure that is causing toxicity, treatment of cells with peptides that do not fold into secondary structures under normal physiological conditions, but that were aberrantly folded into β-sheet structures caused significant toxicity [20].

Therefore, the aim of this study was to establish, given the similarities between human amylin and Aβ, whether human amylin exerts similar neurotoxic effects as shown by Aβ. In addition, cell type specificity of toxicity was also explored by comparing cortical to hippocampal cultures. Aged peptide preparations were compared to non-aged preparations to

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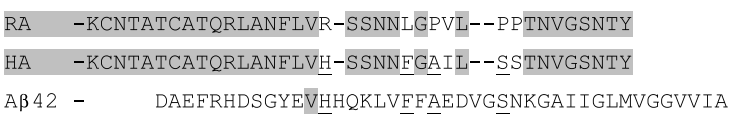


Fig. 1. Alignment of the sequences of human (hu) and rat amylin and comparison to Aβ42. Alignment of rat amylin (RA), human amylin (HA) and Aβ42. Grey shading shows similarities in the rat and human amylin sequences while important similarities between HA and Aβ42 are shown underlined.

ascertain the role of higher order oligomers as toxic agents. Finally, co-incubations of human amylin and Aβ42 were performed to elucidate whether the toxic effects are additive (suggestive of different mechanisms) or not (by using the same pathway).

## 2. Methods and materials

### 2.1. Primary cortical cultures

Primary cortical neuronal cultures were prepared from 16.5-day-old (E16.5) mouse embryos [21]. In brief, embryonic cultures were obtained by sacrificing time-mated C57Bl/6 female mice by cervical dislocation, dissecting the brains, and removing the meninges from the cortices. Cortical tissue was chopped up with a sterile razor blade into small pieces (<1 mm) and transferred to a tube containing 0.025% (w/v) trypsin (Sigma) in Hank Banks' Salt Solution (HBSS, Sigma). After incubation for 15 minutes at 37 °C, DNase (Sigma) was added at 0.0008% (w/v) and the preparation was gently shaken, followed by centrifugation at 720×*g* for 3 m at room temperature (RT). The supernatant was aspirated and the cortical tissue triturated 30 times in plating media consisting of 10% FBS in DMEM using a plugged 1 ml autoclaved pipette tip which resulted in a single cell suspension. Cells were centrifuged at 720×*g* for 3 min at RT, the supernatant was removed and the cell pellet resuspended in plating media. Total cell numbers were determined using a Neubauer haemocytometer (Marienfeld, Germany).

Cortical neurons were plated at a density of 30 000 cells per cover slip on 12 mm glass cover-slips in plating medium, which were precoated with poly-L-lysine (Sigma). The coverslips were washed once with PBS before cells were plated. The cultures were placed in a 5% $CO_2$ incubator at 37 °C for 2 h to allow the cortical neurons to adhere to the coverslips. Then, the plating medium was aspirated and cultures cultivated for 20 days in Neurobasal medium supplemented with 1% (v/v) B27 supplements (Gibco), 0.01% (v/v) 200 mM L-glutamine (Gibco) to minimize growth of microglia and astrocytes. A 50% medium change was performed once every week.

### 2.2. Primary hippocampal cultures

Isolation and preparation of hippocampal cells were done similar to the cortical cell preparations described above, with a few modifications. Hippocampal cell cultures were prepared from 16.5-day-old embryonic (E16.5) mice. Whole brains were isolated and meninges removed as described above. In addition, the opaque horse shoe shaped hippocampal region in each cortex was dissected using fine forceps and single cell suspension was prepared and plated using the same protocol as above. The cultures were placed in a 5% $CO_2$ incubator at 37 °C for 2 h to allow the hippocampal neurons to adhere to the coverslips. Then, the plating medium was aspirated and cultures cultivated for 20 days in Neurobasal medium supplemented with 1% (v/v) B27 supplements (Gibco), 0.25% (v/v) 200 mM L-glutamine (Gibco) to minimize growth of microglia and astrocytes. A 50% medium change was performed once every week.

### 2.3. Preparation of amyloid peptides

Aβ42, rat and human amylin were purchased from Bachem (Germany) (H-9475 and H-7905, respectively). All chemicals were purchased from Sigma unless otherwise stated. Aβ42 was dissolved in sterile PBS to a final concentration of 250 μM and stored at −80 °C until use [22]. Human amylin and rat amylin were dissolved in 0.01 M acetic acid according to the manufacturer's recommendations to final concentrations of 512 μM and 510 μM, respectively, and stored at −80 °C until use. For the mock treatment the same final concentration of acetic acid was used as for the amylin treatments. Peptides used straight from the freezer are non-aged preparations. Aged peptides were prepared by shaking them at 1000 rpm for 24 h at 37 °C to allow the formation of fibrils.

### 2.4. Immunocytochemistry

Hippocampal and cortical neurons were treated after 20 days in vitro (DIV). Aβ stocks were diluted in Neurobasal medium (Gibco) supplemented with 1% (v/v) B27 supplements (Gibco), and 0.25% (v/v) 200 mM L-glutamine (Gibco), at final concentrations of 5 μM and 0.5 μM. Incubations were done for 4 days and all experiments were done in triplicates.

To count the neurons following the incubations, we washed the cells once with pre-warmed PBS to remove excess peptides and fixed them with 4% paraformaldehyde for 10 min at RT [23]. MAP2a + 2b antibody (Sigma, 1:500) specific for MAP2 was used to identify neurons. As a secondary antibody, Alexa-fluor® 555 was used (Invitrogen, 1:250). DAPI was used to identify nuclei. All MAP-2 positive cells and DAPI-positive nuclei in random, non-overlapping visual fields were counted. Then, the ratio was calculated. Finally, all ratios were normalized to that of vehicle treatment and expressed as percentage. MAP2-positive cells were counted blinded by two investigators. Data are shown as means ± standard error of the mean (S.E.M). Statistical comparisons between groups were performed using ANOVA and post hoc tests where conducted using SPSS version 11.

## 3. Results and discussion

### 3.1. Peptide preparation and neuronal cultures

To determine cell-type-specific toxicity of human and rat amylin compared to human Aβ42, we treated cortical and hippocampal cultures with these peptides. Furthermore, we used non-aged and aged peptide preparations as it is well established that aging of amyloidogenic peptides for 24 h at 37 °C promotes aggregation which can contribute to toxicity. Peptide preparations were analysed on a 4–16% gradient tricine gel. Coomassie staining revealed that, different from rat amylin, both the Aβ42 and human amylin preparations contained high order aggregates which did not enter the stacking gel (data not shown).

We prepared both cortical and hippocampal neuronal cultures. After 20 days in culture (20 DIV), the cells were fixed and stained with drebrin to determine whether the cultures were fully differentiated. Staining revealed the presence of dendritic spines in both types of cultures, an indication of terminal neuronal differentiation and thus, the possibility of comparing the two cultures with respect to amyloid toxicity (Fig. 2A). Representative pictures of a healthy and a non-viable neuron are shown (Fig. 2B).

### 3.2. Amyloid toxicity in hippocampal neurons

Toxicity of human amylin towards islet β-cells has been shown to depend on the aggregation state of amylin [24]. Based on the aggregation state of rat and human amylin as well as human Aβ42, we expected that human amylin and Aβ42

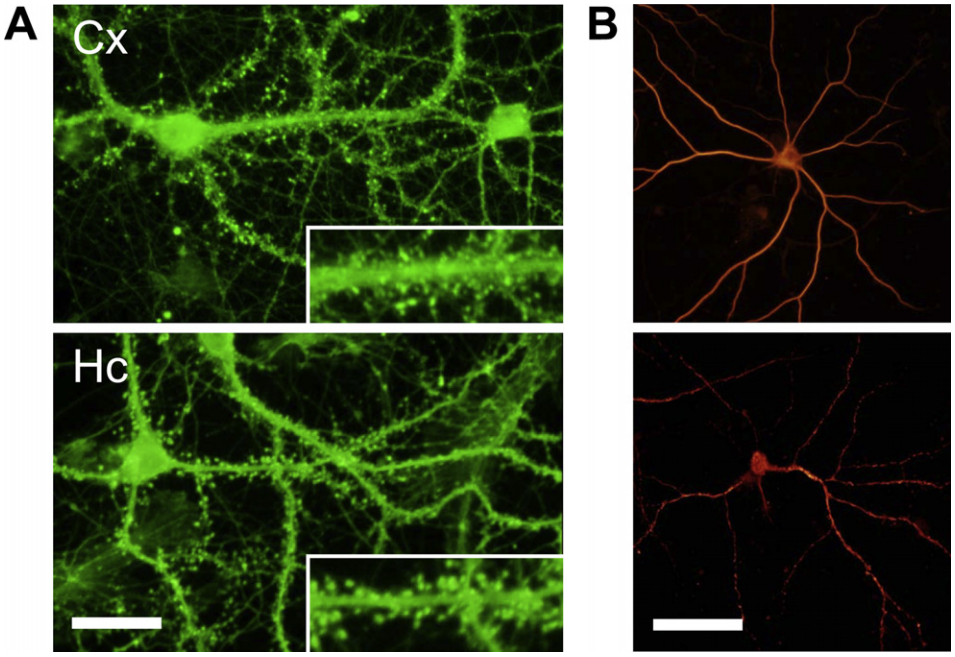


Fig. 2. (A) Primary cortical (Cx) and hippocampal (Hc) neuronal cultures stained for drebrin after 20 DIV (days in vitro). The insets show the dendritic spines of the terminally differentiated neurons at a higher magnification. (B) A healthy (top) and a non-viable (bottom) neuron stained for MAP2. Scale bar: 30 μm (A), 50 μm (B).

would exert a similar pattern of toxicity. This was indeed the case when 20 DIV hippocampal cultures were treated with 0.5 μM and 5 μM preparations for 3, 24, and 96 h (Fig. 3).

At 96 h, non-aged Aβ42 was able to elicit toxicity at 5 μM and this effect was enhanced by aging the peptide that exerted toxicity already at 0.5 μM (Fig. 3A and B). A similar dose-dependency was also observed for human amylin, which showed an enhanced neurotoxicity when the peptide was aged compared to the non-aged preparation (Fig. 3C and D). Acetic acid, which was used as solvent for amylin, did not exert toxicity. Although human amylin was more toxic than Aβ, we found a similar time-dependency under our experimental conditions.

In contrast to human amylin and Aβ42, rat amylin showed either no or very little toxicity to hippocampal neurons at the concentrations and time points tested (Fig. 3E and F). This is consistent with studies in HeLa and β-cell lines where human, but not mouse amylin (which is similar to rat amylin) has been shown to induce aggregation state-dependent apoptosis [25].

Together, as aging of rat amylin did not render it neurotoxic, this suggests that a specific receptor–peptide interaction may be involved in neurotoxicity. Rat amylin (RA) has been shown before to form random globulars, and if this random change in conformation does not elicit toxicity, then toxicity has to be elicited by a specific change in conformation. Furthermore, aging of human amylin and Aβ42, both of which are known to form extended fibrils, causes enhanced toxicity in hippocampal neurons. This suggests that the toxic species under our experimental conditions are probably higher order polymers, rather than monomers.

### 3.3. Amyloid toxicity in cortical neurons

Selective vulnerability has been reported in AD [26]. Specifically, hippocampal neurons are the first to degenerate during AD before other brain areas are affected. To investigate whether this is also reflected by our system, we compared amyloid toxicity between hippocampal and cortical cultures. We found that, different from hippocampal cultures, cortical neurons were generally less susceptible to peptide-induced toxicity (Fig. 4). Aging Aβ42 caused an earlier onset of toxicity (Fig. 4A and B), which was also observed in the hippocampal cultures. (Fig. 3A and B).

Similar to Aβ42, aging of human amylin also enhanced its neurotoxic properties (Fig. 4C and D); similar to what was found for hippocampal cultures.

The above findings reinforce the idea that β-sheet structures are important in conferring toxicity to amyloid, and more importantly, that AD and Type 2 DM are conformational diseases. Toxicity may correlate with the propensity of the peptide to aggregate because the percentage of surviving neurons after treatment with human amylin (HA), which aggregates much faster than Aβ42, was uniformly lower across all treatment groups (for both hippocampal and cortical neurons) than after treatment with Aβ42.

The surprising finding was that 0.5 μM of aged rat amylin was toxic to cortical neurons as early as 24 h. Rat amylin is known not to form fibrils, and aging of this peptide will only lead to random globular structures. Thus, toxicity may be attributed to an unspecific interaction to a receptor found selectively in the cortical neurons but this has to be further investigated.

### 3.4. Combined amyloid toxicity in hippocampal and cortical neuronal cultures

Hippocampal neurons are generally more sensitive to toxic insults. This was also found upon incubation of hippocampal and cortical neurons with staurosporine, a non-selective protein kinase inhibitor that exerts its toxicity, in parts, by interfering with multiple signalling pathways. We found that

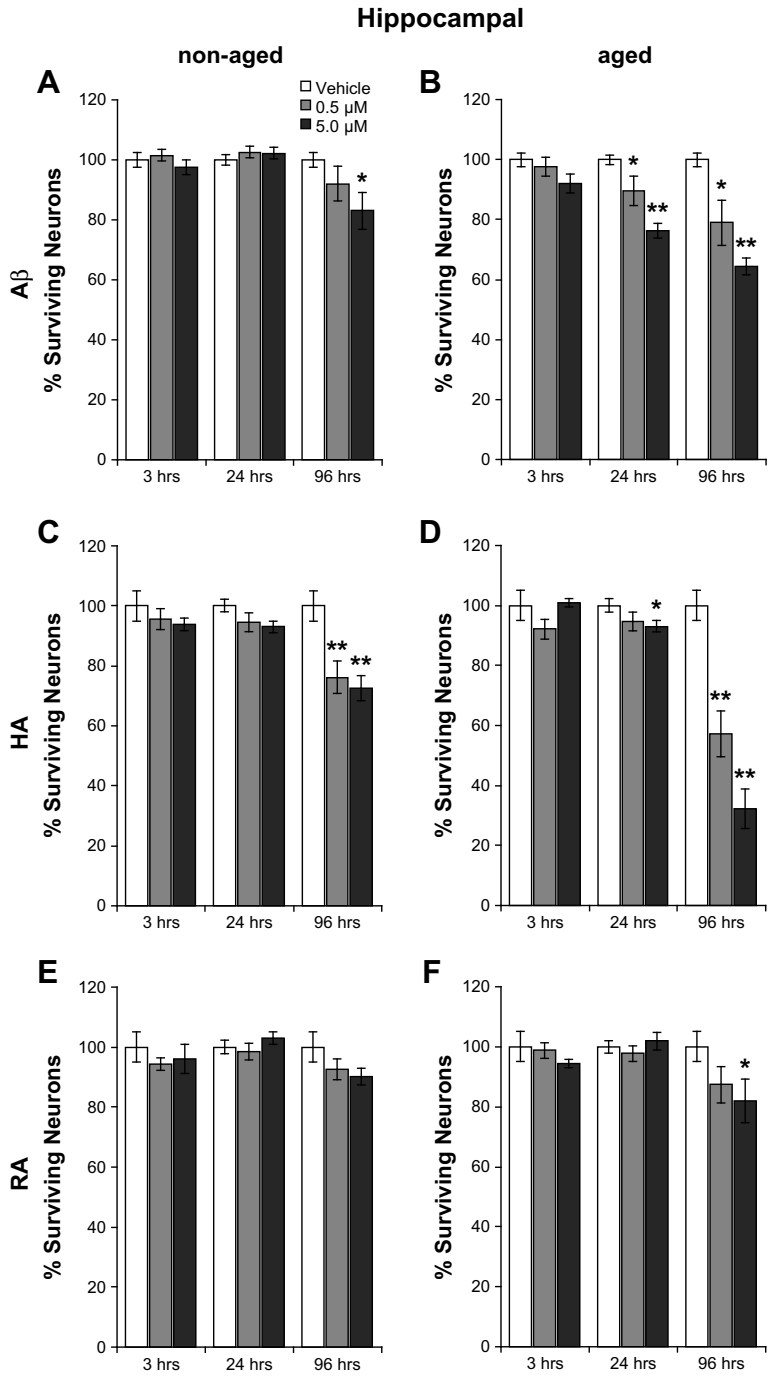


Fig. 3. Treatment of 20 DIV hippocampal neurons with non-aged and aged peptides for 3, 24, and 96 h. (A) Aβ42 exerts a dose-dependent toxicity on neurons which is enhanced upon aging. (B) HA also exerts a dose-dependent toxicity on neurons, like Aβ42, and is enhanced upon aging of HA. (C) RA is virtually not toxic to hippocampal neurons. All values are normalized against their respective vehicles and error bars represent the S.E.M. $^{*}P < 0.05$, $^{**}P < 0.001$.

toxicity was much more pronounced with staurosporine than with either Aβ or human amylin (with up to 72% cell death) (data not shown). As expected, hippocampal neurons were more sensitive than cortical neurons, possibly reflecting the generally higher content of supportive glia in cortical cultures.

Aspects of Aβ toxicity can be blocked in rat forebrain primary cultures using human amylin antagonists [26]. It has also been suggested that Aβ42 and human amylin may act on the same receptor [16]. To investigate this possibility in our system, we incubated hippocampal and cortical cultures with either non-aged or aged Aβ, human amylin or a combination of both peptides for 24 h. We found that co-incubation of hippocampal cultures with 5 μM non-aged Aβ42 and 5 μM non-aged human amylin did not enhance toxicity compared to single treatments (Fig. 5A), suggesting that the two peptides may possibly act on the same pathway. In contrast, cortical neurons showed an enhanced toxicity upon co-incubation (Fig. 5A).

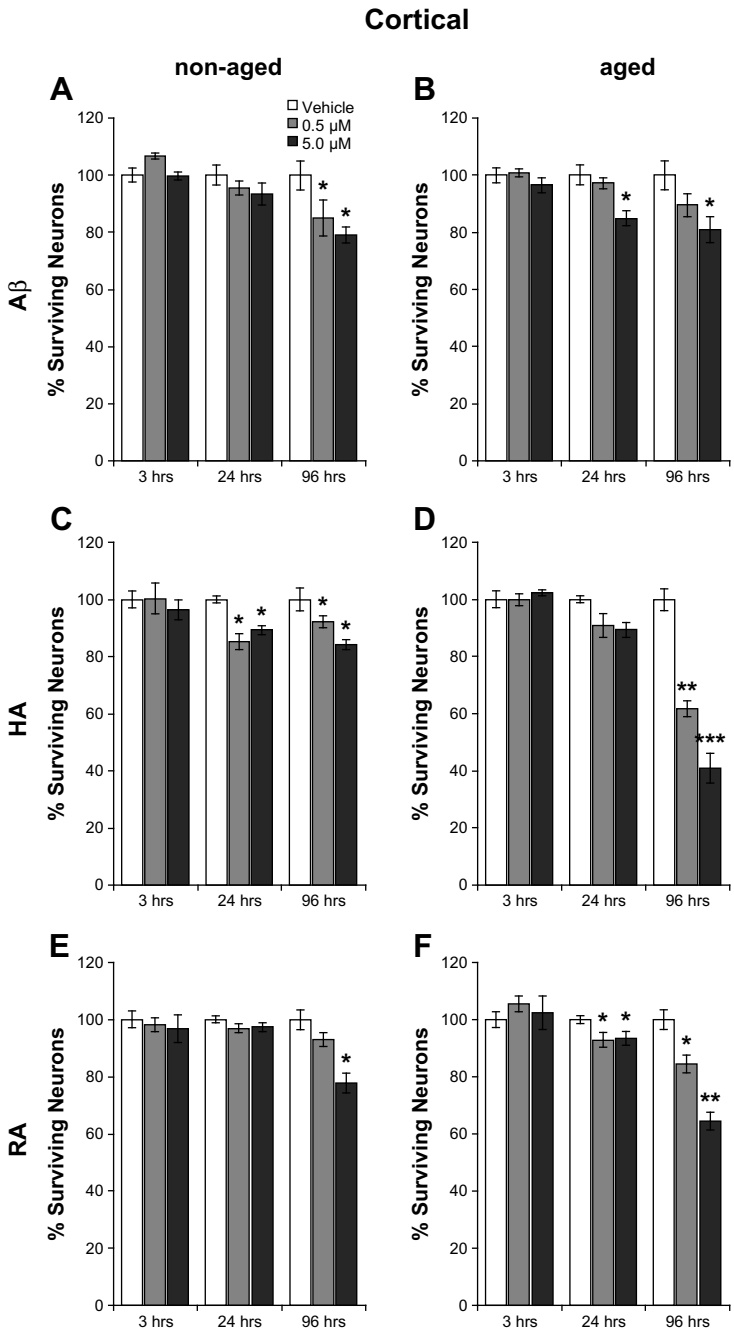


Fig. 4. Treatment of 20 DIV cortical neurons with non-aged and aged peptides for 3, 24, and 96 h. (A) Aβ42 exerts a dose-dependent toxicity on neurons which is enhanced upon aging of Aβ42, like in the hippocampal cultures. The survival rate of the cortical cultures are higher than hippocampal cultures. (B) HA also exerts a dose-dependent toxicity on neurons, like Aβ42, and this is enhanced upon aging of HA. (C) RA is toxic to cortical neurons but the effect was not enhanced upon aging of RA. All values are normalized against their respective vehicles and error bars represent S.E.M. $^{*}P < 0.05$, $^{**}P < 0.001$.

This suggests the possibility of more than one pathway in these neurons in mediating toxicity of Aβ42 and human amylin.

When we treated either hippocampal or cortical neurons with aged peptides, human amylin alone already exerted a very pronounced toxicity that was not further increased by Aβ, suggesting that this highly toxic nature of human amylin masks any toxicity effects of Aβ42 (Fig. 5B).

In conclusion, we found that aggregation of Aβ42 and human amylin enhanced their toxicity effects uniformly across both hippocampal and cortical cultures. This effect was not observed in cultures treated with rat amylin. We observed that in general, hippocampal neurons were more sensitive than cortical neurons to toxicity exerted by either human amylin or Aβ42. We also found that rat amylin, which does not form fibrils, is virtually not toxic to hippocampal neurons but surprisingly, it exerted toxicity in cortical neurons in our system. Furthermore, co-incubation of Aβ42 and human amylin suggests the possibility of a shared pathway in hippocampal

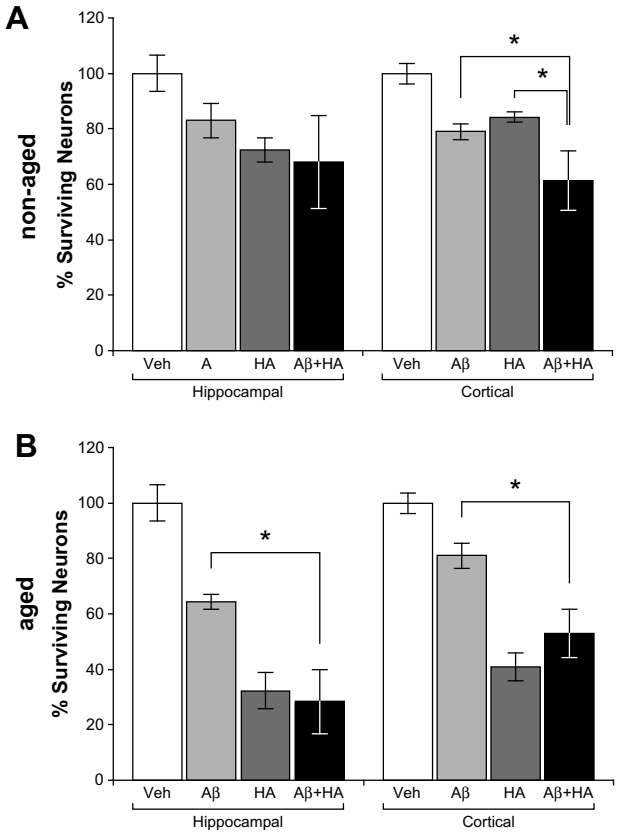


Fig. 5. Combined amyloid toxicity in hippocampal and cortical neuronal cultures treated for 24 h with 5 μM aged preparations of peptides. (A) Treatment of hippocampal and cortical neurons with Aβ and HA, either alone or in combination. Treatment of hippocampal neurons suggests that HA and Aβ use the same receptor or pathway to exert toxicity whereas in cortical neurons they may use different receptors and/or pathways. (B) When using aged preparations, the pronounced toxicity of human amylin likely masks that of Aβ precluding a conclusion as to whether the effects of the two peptides are additive or not. All values are normalized against their respective vehicles and error bars represent S.E.M. $^{*}P < 0.05$, $^{**}P < 0.001$.

neurons whereas more than one pathway may be activated in cortical neurons. In future studies, antagonists may be utilized to discriminate these possibilities. Our results suggest that specific conformational changes in peptides are important determinants of toxicity. This notion is supported by the finding that conformational antibodies can be generated that recognize a generic amyloid fibril epitope that is found, among others, on amyloid-like aggregates derived from proteins such as transthyretin, amylin or β2-microglobulin [27]. Further studies involving expression of human and rat amylin in vivo could aid in elucidating these mechanisms and possible interactions to inhibit misfolding of proteins in diseases such as AD and DM [13,24].

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